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ON THE ORIGIN OF A FACTOR CAUSING DEPROTEINIZATION OF VACCINIA VIRUS NUCLEOCAPSID

[Following is the translation of an article by L.A. Melnikova, I.A. Koslova, O.P. Bergerson and V.M. Zhdanov, Institute of Virology imeni D.I. Ivanovskogo, AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), 1965, No. 4, pp 447-453. It was submitted on 14 August 1964. Translation performed by Sp/7 Charles T. Osterlag, Jr.]

A peculiarity of the intracellular development of viruses from the variola group, differentiating them from other viruses, is the two-phase process of virion deproteinization and the liberation of viral nucleic acid from the protein membrane. The first phase is carried out in virus sensitive cells immediately following the penetration of virus particles by means of viropexis: This phase is completed in vacuoles by the liberation of nucleocapsid from the external membranes and apparently is guaranteed by enzymes from the cell wall. The second phase is preceded by the resorption of the vacuole and the discharge of the nucleocapsid into the cytoplasm of the cell. After this a factor appears in the cell which ensures the final deproteinization of virus DNA [7].

Up until now the nature and origin of this factor have remained unclear. During inactivation of the virus by various agents which damage protein, for example by heating, this factor is not formed and deproteinization of the virus ceases in the first phase. On the other hand the addition, to cells infected with such a virus, of living viruses from the variola group or the same viruses with impaired nucleic acid (treated with mustard gas) leads to the formation of this factor and the completion of deproteinization of the virions which had penetrated into the cell [5]. In connection with this, the in-cell formation of the factor causing the deproteinization of the nucleocapsid of viruses from the variola group is a necessary phase in the processes of multiple reactivation and recombination of these viruses [1,2].

Apparently the factor causing the deproteinization of the nucleocapsid of viruses in the variola group has an enzymatic nature. However, up until recently it has remained unclear whether this enzyme is coded in the virus or if the virus is only an inducer of this factor, coded in the cell.

In the present paper an attempt is made to answer this question. The methods of our investigations are somewhat different than in recently published papers [3].

Materials and Methods

Cells. In the experiments we used a 2-day culture of chick fibroblast cells, incubated in test tubes in medium No. 199 with the addition of 5% bovine serum.

Viruses. The investigations were carried out with smallpox vaccine viruses (dermovaccine strain) and cowpox virus (Brighton strain). The smallpox vaccine virus was introduced into the cell culture in a quantity of 5-10 PFU per one cell.

For inactivation we used the smallpox vaccine virus, obtained by means of centrifugation of a suspension of infected chorio-allantois membranes at 30,000 rpm for one hour and resuspended in a phosphate buffer with pH 7.2-7.4. The titer of the virus was 10^{-7} ID. The virus was heated in test tubes in a volume of 1 ml for 2½-3 hours at 56°. Prior to infection the virus was diluted by 10 times. The absence of infectivity in the virus was determined by means of a two-fold passage on the chorio-allantois membranes of chick embryos.

As the reactivator we selected the cowpox virus, which was introduced into the cell culture also in a dose of 5-10 PFU for 2½-3 hours before their infection with heated virus.

The infected cells were investigated in 1, 2, 4, 6, 8, 10, 12, and 14 hours after infection. The presence of virus was determined after a 3-fold freezing and thawing out of infected chorio-allantois membranes of 12-day old chick embryos, which were then investigated after 48 hours following infection for the presence of plaques and by titration in the hemagglutination reaction.

The cellular extracts were prepared from the cells of normal chicken fibroblasts and from these infected with smallpox vaccine virus. A two-day culture of chick fibroblastic cells, incubated in flasks in Medium No. 199, was infected with the smallpox vaccine virus in a dose of 10 PFU per one cell for 2-2½ hours. Then the nonadsorbed virus was removed and the cells thoroughly washed with physiological solution and neutralized with antivariolous serum. The cells were removed mechanically, precipitated by centrifugation at 3000 rpm for 15 minutes, then centrifuged twice at 2200 rpm. The precipitate was homogenized in a Potter homogenizer and resuspended in medium No. 199 up to 10% concentration. In the same manner we prepared the extracts from cells, treated with actinomycin D in a dose of 0.05 γ/ml for 18-24 hours before their infection with virus. In the experiments we used only those extracts which did not allow the growth of virus during a two-fold passaging on the chorio-allantois membrane of chick embryos. The extracts and the virus were introduced at the same time and in the same quantity (0.1 ml).

Puromycin was introduced into a culture of chick fibroblast cells in a dose of 20-80 γ/ml also simultaneously with the virus 2 and 3 hours before infection. After a one hour contact with the cells the virus was washed off with physiological solution and specific serum. The culture, washed of nonadsorbed virus, was flushed with nutrient medium containing puromycin in the same doses.

Results

The results of the investigations, conducted with live intact virus and virus inactivated by means of heating, are presented in Tables 1 and 2.

Under the conditions of our experiments mature virus was detected in 7 hours after infection with live intact virus; of course during infection with heated virus mature virus did not appear. The addition of extracts from cells infected with the same virus in 2-2½ hours after inoculation, reduced the latent period by 3 hours and the mature virus appeared in 4 hours following infection. The periods for the appearance of mature virus were the same during infection both with live and heated virus. This indicates that reactivation processes took place in the latter case. It should be noted that the extracts alone, obtained in the first 2-2½ hours after infection of the cells, as already mentioned, did not contain virus. This excludes the possibility of even individual virus particles entering the cultures. On the other hand, the extracts from normal tissues in experiments with live virus did not reduce the periods for the appearance of the mature virus, and in tests with inactivated virus did not lead to its reactivation. Consequently, the shortening of the latent period was connected with the presence of infected cells in the extract, that is the factor guaranteeing the outbreak of virus infection, in other words, the factor completing the deproteinization of the virus particle.

Completely analogous results were obtained when the cells were preliminarily infected with another virus of the variola group (cowpox virus). In contrast to the smallpox vaccine virus, which causes the formation of large white plaques, the cowpox virus forms small red plaques, therefore both viruses are easily differentiated on chorio-allantois membrane. Mature particles appear in 4 hours after infection with smallpox vaccine virus, that is, 3 hours earlier than in the control, both with live and with heated virus.

For clearing up the problem concerning the origin of the factor under study tests were set up with actinomycin D, taking into consideration that this antibiotic reacts specifically with the double thread structures of DNA, preventing the formation of cellular information RNA, necessary for the synthesis of protein.

The preliminary treatment of cell cultures with actinomycin D in a dose of 0.05 γ/ml sharply inhibited the development of smallpox vaccine virus: The mature virus was detected only after 12 hours, that is, 4 hours later than in the tests without actinomycin. These data differ with the results of recently published papers [4]. They testify to the almost complete suppression of virus multiplication at a dose of 0.1 γ/ml. This can be explained by the low concentration of actinomycin in our experiments and probably by the gradual destruction of the latter in the cells. The extracts of cells, infected with virus in the presence of actinomycin, did not reduce the latent period of smallpox virus development, and also did not prevent its development. Consequently, they do not contain the deproteinization factor for smallpox virus.

Therefore, the conclusion can be made that the genetic information concerning this factor is contained in the cellular and not the virus genome. This conclusion is also supported by the fact that extracts of infected cells, added to heated virus, caused the reactivation of this virus in cells treated with actinomycin. This is very clearly confirmed in experiments in which the cells were treated with an extract from cells treated with actinomycin D and infected with smallpox vaccine virus.

Under such an experimental set-up the reactivation of heated virus did not occur.

For clarification of the nature of the deproteinization factor for the nucleocapsid of smallpox vaccine virus, and also of the role of the cell in this process, we used puromycin as the inhibitor of protein synthesis.

In Series I of the experiments the dose of puromycin was 20 γ/ml. When the cells were treated with that quantity of puromycin there was a sharp increase in the latent period of development of the smallpox vaccine virus; the mature virus appeared after 10 hours, that is, 3 hours later than in the control. If the same virus was inoculated in cells treated with puromycin and an extract of infected cells added, then the latent period was sharply reduced and the puromycin did not stop the development of virus infection. In the experiments with inactivated virus the reduction in the length of the latent period was less expressed. In general, the results of these tests are similar to the results of the tests with actinomycin, though in this case protein synthesis was inhibited. The development of the virus in the presence of puromycin may be explained by the fact that the dose of it was not great and could therefore only restrain, but not completely block, the synthesis of viral proteins, acting much more strongly on the synthesis of cellular proteins.

When the cells were treated with larger doses of puromycin (80 γ/ml) we noted the complete suppression in the synthesis of smallpox vaccine virus in investigations 24 hours after infection.

In Table 3 we present the results of the 3 experiments on the influence of puromycin on the reactivation of heated virus. As it seen from these data, treating the cells with puromycin simultaneously or 2 and 3 hours prior to the administration of heated virus and the extract from cells infected with live virus prevents the reactivation of this virus.

These data testify that puromycin not only suppresses the synthesis of cellular protein, but also influences the factor of nucleocapsid deproteinization, which makes it possible to express the supposition concerning its protein nature.

In addition to this we carried out supplementary investigations on the study of the action of puromycin on the multiplication of smallpox vaccine virus and the synthesis of the factor causing deproteinization. Taking into consideration the data from the literature [2,7] and our data that this factor is formed in early periods following infection, puromycin [6] was introduced in the first hours after infection and with 30 minute intervals (Figure 1). The results of these tests showed that when puromycin was administered 30 minutes after infection, the complete suppression of virus synthesis was noted when the investigation was made 24 hours after infection. When puromycin was administered 1-1½ hours after infection the virus was detected in a titer of 10^{-1} ID, after 2-2½ hours the titer of the virus in the test group of cells comprised 10^{-2} ID, and then increased up to 10^{-3} ID. At the same time in the control the titer of the virus was 10^{-7}. The results of these tests again urge that puromycin suppresses the synthesis of smallpox vaccine virus, and the complete suppression of virus multiplication 30 minutes after infection creates a foundation for the proposal that the factor under study has a protein nature and is formed during the early periods of infection.

For a more accurate quantitative characterization of the dynamics in the multiplication of the smallpox vaccine virus we set up tests with the titration of the virus on chorio-allantois membrane based on plaques and on the hemagglutination reaction (the reaction was used only with live intact virus).

In figures 2 and 3 we present the data from the tests with live intact virus. As can be seen from these drawings, the addition of extracts from virus infected cells to the culture not only reduces the periods for the appearance of the mature virus, but also increases its titer. This is expressed still more if another virus of this group (cowpox virus) is added to the culture. Treatment of the culture with actinomycin D increases the latent period and lowers the intensity of multiplication. Here the plaque method is more sensitive than the method of virus titration in the hemagglutination reaction. In the latter case the periods for the detection of the virus are drawn back by 2 hours (Figure 4).

In the tests with the heated virus the addition of extract or heterogeneous smallpox virus leads to the reactivation of this virus. However, its titers are somewhat lower than in the tests with live virus.

Discussion

As is already known, for the synthesis of smallpox vaccine it is necessary that its DNA be liberated. This takes place in 2 phases, while the final deproteinization is accomplished due to a special factor, the nature and origin of which remain unclear up to the present time. There is doubtless interest in the question of whether this factor (enzyme) is coded in the cell or in the virus, or is the latter an inducer of this factor.

For clearing up this problem we made use of antimetabolites: Actinomycin D as an inhibitor of information DNA, necessary for the synthesis of protein, and puromycin, which is a suppressor of protein synthesis. The well studied phenomenon of reactivation served as the criterium for the deproteinization of viral nucleocapsid and the liberation of viral DNA. Simultaneously with heated virus, analogous variants of the experiments were carried out with live intact virus. The reactivation of heated virus was successfully reproduced not only with native cowpox virus, but also with extracts obtained from cells infected with smallpox vaccine virus. These data conform to the results of recently published papers in which it was demonstrated that extracts, obtained from cells of chick fibroblasts infected with native cowpox virus, are capable of reactivating heated virus already in 2 hours after infection. The maximum reactivating activity was possessed by cellular extracts obtained 3-6 hours after infection. It was shown earlier [7] that the reactivating factor, investigated based on the sensitivity of DNA to DNAase, is manifested considerably earlier. The extracts from infected cells in the tests with live virus act the same as the cowpox virus, that is, they shorten the period of virus initiation by 3 hours. All of this points to the fact that in the infected cells there is a factor causing the liberation of viral nucleic acid and the subsequent synthesis of mature virus particles. However, as it was already stated, it remains unclear if this factor is coded in the cell or in the virus, or if it is induced in the cell under the influence of the virus. Light was shed on this problem by data from various experiments carried out with actinomycin D, which we used as the suppressor of information RNA; consequently the normal information for the synthesis of protein was disrupted in treated cells.

The fact that extracts from cells treated with actinomycin D and infected with live smallpox vaccine virus (period of infection 2½ hours) did not cause the phenomenon of reactivation of heated virus, and did not reduce the period of initiation for native virus, speaks in favor of the following: The factor under study, ensuring the deproteinization of the

nucleocapsid of the smallpox vaccine virus, is coded in the genome of the cell and not of the virus. This proposition is also supported by the experiments in which the preliminary treatment of the cells with actinomycin D did not prevent the reactivation of heated virus following the addition to the cells of the prepared factor contained in the extract of infected cells.

The purpose of the experiments with puromycin was to clear up the nature of the factor under study. A puromycin dose of 20 γ/ml had almost no influence on the multiplication of live virus and did not inhibit the reactivation of heated virus following the addition of extracts from infected cells. However, when the cells were treated with a large dose of puromycin (80 γ/ml) the synthesis of smallpox vaccine virus was completely blocked and reactivation of heated virus was prevented even with the addition of an extract of infected cells.

The data obtained testifies to the protein nature of this factor.

Conclusions

1. In the early stages of cell infection with the smallpox vaccine virus (2-2½ hours) a factor is formed which ensures the deproteinization of the virus nucleocapsid.

2. This factor also ensures the possibility of the reactivation of heated virus.

3. The deproteinization factor has a protein nature and is coded in the genome of the cell-host.

Literature

1. Chernos, V.I., Gendon, Yu. Z., Vopr. virusol., 1964, No. 2, p 148.

2. Abel, P., Z. Verebungsl., 1963, Bd 94, S 249.

3. Fenner, F., Holmes, I.H., Joklik, W.K., et al., Nature, 1959, v 183, p 1340.

4. Hanafusa, T., Kamahora, J., Hanafusa, H., Virology, 1959, v 8, p 525.

5. Joklik, W.K., et al., Nature, 1960, v 186, p 992.

6. Joklik, W.K., Virology, 1964, v 22, p 620.

7. Idem., J. molec. Biol., 1964, v 8, p 263.

8. Shatkin, A.J., Nature, 1963, v 199, p 357.

Table 1

Periods for the appearance of mature virus in tests with live intact smallpox vaccine virus

Test conditions	Time after infection (in hours)								
	2	3	4	5	6	7	8	10	12
Smallpox vaccine virus(5-10 PFU for one cell) .						+	+	+	+
Extract from infected cells and smallpox vaccine virus (noninfectious)			+	+	+	+	+	+	+
Extract from normal cells and smallpox vaccine virus					+	+	+	+	+
Extract from actinomycin D treated & infected cells and smallpox vaccine virus							+	+	+
Cowpox virus & smallpox vaccine virus (5-10 PFU for one cell) .			+	+	+	+	+	+	+
Puromycin, extract from infected cells and smallpox vaccine virus			+	+	+	+	+	+	+
Actinomycin D & smallpox vaccine virus								+	+
Puromycin and smallpox vaccine virus								+	+

* In the Russian original the last two entries were dealt with by only one row of symbols approximately between both lines.

Table 2

Periods for the appearance of mature virus in cells with inactivated vaccinia virus.

Test conditions	Time after infection (in hours)								
	2	3	4	5	6	7	8	10	12
Heated smallpox vaccine virus (10^{-7} ID) . . .	-	-	-	-	-	-	-	-	-
Cowpox virus + heated smallpox vaccine virus (10^{-10} ID) . . .	-	-	+	+	+	+	+	+	+
Extract from infected cells & heated smallpox virus (noninfectious)	-	-	+	+	+	+	+	+	+
Extract from normal cells and heated smallpox vaccine virus .	-	-	-	-	-	-	-	-	-
Extract D and heated smallpox vaccine virus (noninfectious)	-	-	-	-	-	-	-	-	-
Actinomycin D, extract from infected cells & heated smallpox vaccine virus			+	+	+	+	+	+	+
Puromycin and extract from infected cells and heated smallpox vaccine virus					+	+	+	-	

Table 3

luence of Puromycin on the reactivation of heated virus

Experiment	Time after infection (in hours)						
	2	4	5	8	10	14	24
Puromycin, extract (noninfectious) from infected cells and heated virus	-	-	-	-	-	-	-
Extract from infected cells and heated virus	-	-	+	+	+	+	
Heated virus (10^{-7} ID up to inactivation)	-	-	-	-	-	-	-

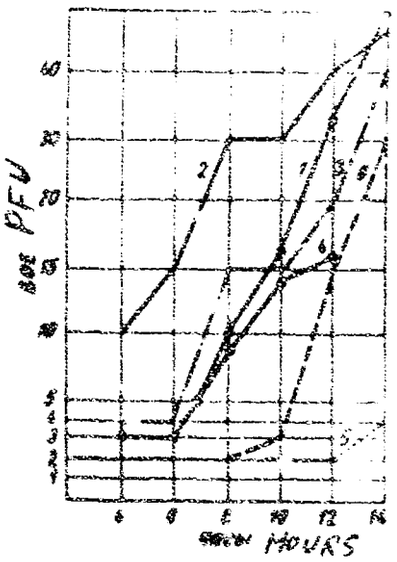

Figure 1. Influence of puromycin on the synthesis of smallpox vaccine virus.

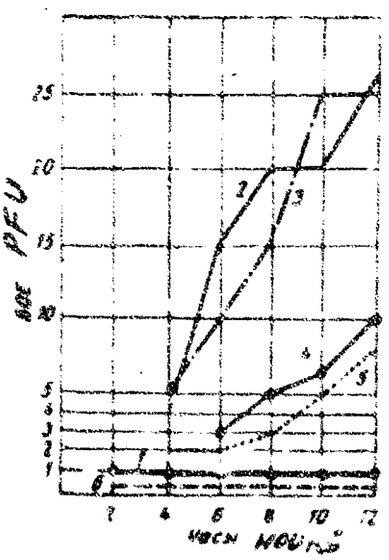

Figure 2. Dynamics of development of live smallpox vaccine virus, measured in PFU;
1 - smallpox vaccine virus; 2 - cowpox virus and smallpox vaccine virus; 3 - extract of cells infected with smallpox vaccine virus and smallpox vaccine virus; 4 - puromycin, extract from cells infected with smallpox vaccine virus and smallpox vaccine virus; 5 - actinomycin D and smallpox vaccine virus; 6 - extract of cells treated with actinomycin D and infected with smallpox vaccine virus, and smallpox vaccine virus.

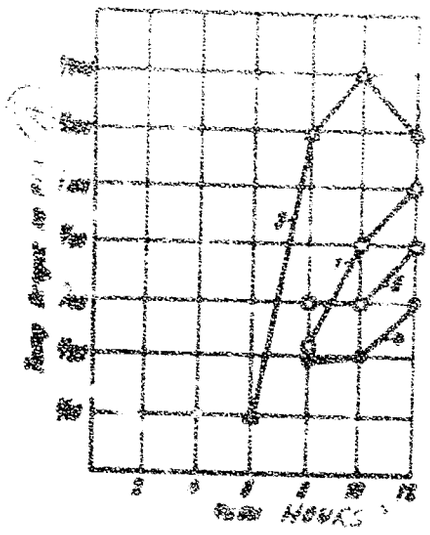

Figure 3. Dynamics of development of inactivated smallpox vaccine virus, measured in PFU. Legend same as in figure 1.
A - titer of virus based on the hemagglutination reaction.

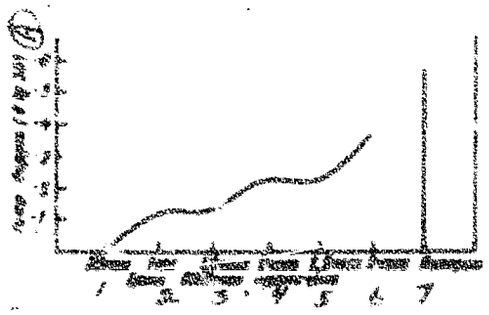

Figure 4. Dynamics of development of live smallpox vaccine virus, measured according to the hemagglutination reaction.
A = Titer of virus (in lg ID)
Periods for the administration of Puromycin: 1 (30 min.); 2 (1 hr); 3 (1,2 hr); 4 (2 hr); 5 (2,2 hr); 6 (3 hr); 7 (control).